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(57) Abstract

The present invention provides replication-defective hybrid retroviral vectors comprising GaLV components and methods for preparing and using such vectors. The vectors comprise an envelope component, a core component and a defective genome, at least one of which is derived from GaLV. The vectors can comprise the minimal *cis* acting sequences from GaLV that allow packaging of the defective genome in a hybrid virion.

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Gibbon Ape Leukemia Virus-based Retroviral Vectors

BACKGROUND OF THE INVENTION

The present invention relates generally to retroviral vectors. In particular, the invention relates to retroviral vectors comprising nucleic acid sequences from Gibbon Ape Leukemia Virus.

Considerable effort is now being directed to introducing engineered genes into mammalian cells for a variety of applications including gene therapy and the production of transgenic animals. Such strategies are dependent upon the development of effective means for safe delivery of genes to appropriate target cells and tissues.

Retroviral vectors are particularly useful for directing desired polynucleotides to the appropriate cells and integration of the polynucleotides in the host cell genome. For example, the majority of the approved gene transfer trials in the United States rely on replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (Miller et al. *Mol. Cell. Biol.* 10:4239 (1990); Kolberg R *J. NIH Res.* 4:43 (1992); Cornetta et al. *Hum. Gene Ther.* 2:215 (1991)). As is known in the art, the major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer into certain types of replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transfer.

Unfortunately, many human cells are not efficiently infected by prior art retroviral vectors. Reduced susceptibility to retroviral infection is most likely due to inefficiencies in one of three stages of viral replication: 1) binding to retroviral receptors on the cell surface and early viral entry, 2) late entry and transport of the viral genome to the cell nucleus and integration of the viral genome into the target cell DNA, and 3) expression of the viral

genome. These three stages are governed, respectively, by the viral envelope proteins, the viral core proteins, and the viral genome. All three of these components must function efficiently in a target cell to achieve optimal therapeutic gene delivery.

Gibbon Ape Leukemia Virus (GaLV) uses a cell surface internalization receptor that is different from those of the available retroviral vectors and thus allows infection of cells and tissues normally resistant to retroviral infection. The human receptor for GaLV has recently been cloned and shows a wide cell type and species distribution. Johann et al., *J. Virol.* 66:1635-1640 (1992). Indeed, GaLV can infect many mammalian species with the notable exception of mouse cells. The same receptor is used by simian sarcoma associated virus (SSAV), a strain of GaLV. Sommerfelt et al., *Virol.* 176:58-59 (1990).

The construction of hybrid virions having GaLV envelope proteins has been demonstrated. For instance, Wilson et al., *J. Virol.* 63:2374-2378 (1989), describe preparation of infectious hybrid virions with GaLV and human T-cell leukemia virus retroviral *env* glycoproteins and the *gag* and *pol* proteins of the Moloney murine leukemia virus (MoMLV). In addition, Miller et al., *J. Virol.* 65:2220-2224 (1991), describe construction of hybrid packaging cell lines that express GaLV envelope and MoMLV *gag-pol* proteins.

Existent retroviral vectors capable of infecting human cells all contain core and genome components that derive from MoMLV. For human cells which are resistant to efficient infection by such vectors at any of the three stages noted above, new vectors comprising improved envelope, core or regulatory sequences must be designed. Thus, there is a need to design retroviral vectors components which can be used to introduce genes into human cells not efficiently infected by the currently utilized retroviral vectors. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides recombinant DNA constructs comprising a defective viral genome having a polynucleotide sequence of interest and a GaLV component. For instance, the GaLV component may be a GaLV packaging site which directs packaging of the defective viral genome in an infectious, replication-defective virion. The packaging site typically consists of between about 150 base pairs and about 1500 base pairs and includes a sequence extending from about position 200 to about position 1290 of the sequence shown in Figure 1.

The construct may further comprise GaLV regulatory sequences which direct expression of the polynucleotide of interest. Typically, the regulatory sequences comprise a GaLV (e.g., GaLV SEATO or GaLV SF) 5' or 3' LTR promoter.

The invention also relates to mammalian cells comprising the defective viral genome described above. The mammalian cells may be packaging cells, in which case the cells will also contain retroviral *gag*, *pol* and *env* genes. These genes may be derived from MoMLV, GaLV SF or GaLV SEATO. Packaging cells conveniently used in the invention include PG13 and PA317.

The invention further provides isolated hybrid virions comprising GaLV (e.g., SF or SEATO) envelope proteins and an RNA genome comprising a polynucleotide sequence of interest and a GaLV component. The virions typically contain GaLV core proteins. MoMLV core proteins can also be used.

The invention also provides isolated recombinant DNA constructs comprising polynucleotide sequences which encode an infectious GaLV virion capable of infecting a mammalian cell and producing functional viral progeny. The infectious clones typically comprise about 97% GaLV SEATO sequences and 3% GaLV SF sequences.

Also disclosed are methods of introducing a polynucleotide of interest into human cells using the hybrid virions described above. The methods are preferably used as part of a gene therapy protocol for treating a human patient.

DEFINITIONS

A "hybrid virion" is a virion comprising genome, core, and envelope components derived from more than one virus. The term specifically includes "pseudovirions" which historically have been defined as containing the genome from one virus and the structural proteins from another.

A "packaging cell" is a genetically constructed mammalian tissue culture cell that produces the necessary viral structural proteins required for packaging. The cells are incapable of producing infectious virions until a defective genome is introduced into the cells. The genetic material for the viral structural proteins is not transferred with the virions produced by the cells, hence the virus cannot replicate.

A "replication-defective" virion or retroviral vector is one produced by a packaging cell as defined above. Such a virion infects a target cell but is incapable of producing progeny virions which can infect other cells.

Two polynucleotides or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues in the two sequences is the same when aligned for maximum correspondence. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection. These references are incorporated herein by reference.

The percentage of sequence identity between two sequences is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the

number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

For instance, a preferred method for comparing sequences uses the GAP program based on the algorithm of Needleman at al., *supra*. Typically, the default values for all parameters are selected. These are gap weight: 5.0, length weight: 0.30, average match: 1.0, and average mismatch: 0.0.

The term "substantial identity" means that a polynucleotide or polypeptide comprises a sequence that has at least 80% sequence identity, preferably 90%, more preferably 95% or more, compared to a reference sequence over a comparison window of about 20 bp to about 2000 bp, typically about 50 to about 1500 bp, usually about 350 bp to about 1200. The values of percent identity are determined using the GAP program, above.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.2 molar at pH 7 and the temperature is at least about 60°C.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete sequence of the GaLV SEATO genome, as published in Delassus, *et al.* (1989) *Virol.* 173:205-213.

Figures 2A-2F show the construction of the infectious GaLV clone of the invention.

Figure 3 shows packagable defective genomes of the present invention.

Figure 4 shows schematic diagrams of plasmids 395, 558, and 521.

Figure 5 shows schematic diagrams of plasmids 395, 559 and 537.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

New hybrid retroviral vectors comprising GaLV components are provided by the present invention. The tissue specificity of the vectors is determined by the viral envelope proteins, the viral core proteins, and the viral genome, at least one of which is derived from GaLV. The vectors can comprise the minimal *cis* acting sequences (packaging signals) from GaLV that allow packaging of a defective genome in a replication-defective hybrid virion. In addition, the LTR of the defective genome can be derived from GaLV. For instance, the 3' LTR region of the hybrid retroviral vector can be selected from various GaLV sequences to provide desried tissue specific expression of the structural genes in the genome.

Replication-defective retroviral vectors are produced when a defective DNA viral genome is introduced into a packaging cell line. The defective genome contains the sequences required for integration into the target cell genome, for packaging of the genome into infectious virions, as well as those viral sequences required for expression of the therapeutic gene or other polynucleotide contained within the defective viral genome. The packaging cells comprise the *gag*, *pol*, and *env* genes which encode the viral core and envelope components. These core and envelope proteins assemble around the defective genome, thus producing retroviral vectors.

A number of standard techniques are used to ensure safety of retroviral vectors. For instance, the defective genome is introduced into the cell separately from the genes encoding the core and envelope components. In this way, recombination between the genome and the core and envelope genes, which would lead to the packaging of complete viral

genomes, is extremely unlikely. The resulting virions should therefore not comprise the *gag*, *pol*, and *env* genes and are thus replication-defective. Homologous recombination, however, between the inserts can lead to the production of infectious virions. Typically, the packaging cells are produced by introducing the *gag*, *pol*, and *env* genes on at least two separate plasmids. This scheme effectively prevents homologous recombination leading to reconstruction of infectious virus because the probability of multiple, independent homologous recombination events occurring is extremely low.

Retroviral vectors can also be designed to prevent synthesis of viral proteins by the integrated defective genome. For instance, if a portion of the *gag* gene is included to increase packaging efficiency, a stop codon can be introduced into the gene to prevent synthesis of *gag* proteins. Miller et al., *BioTechniques* 7:982-988 (1989), which is incorporated herein by reference.

In addition, the cells used to make packaging cells do not possess a cell receptor for GaLV and are thus not infectable by GaLV. Retroviral vector virions having the GaLV envelope therefore cannot reinfect the packaging cells and vector spread in the packaging cells is greatly reduced. Suitable packaging cells also have limited or no endogenous viral sequences. Cell lines for this purpose include the *Mus dunni* tail fibroblast cell line. This strategy decreases the potential for generation of recombinant vectors, which are often transmitted with higher efficiency than the parental vector.

Finally, replication-defective vectors of the invention are particularly safe because GaLV is evolutionarily derived from a xenotropic virus of an asian strain of mouse and does not appear to be closely related to human pathogenic viruses. Thus, in terms of containment, GaLV-based, replication-defective hybrid virions are as safe as prior art murine retroviral vectors and provide a safe vehicle for delivery of genes for human gene therapy.

The packaging cell lines of the invention can be used to provide infectious replication-defective hybrid virions for use in gene transfer in humans, hamsters, cows, cats, dogs, monkeys, chimpanzees, macaques, primates, and other species whose cells have host cell receptors for GaLV envelope proteins.

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and cell culture. Generally, enzymatic reactions, oligonucleotide synthesis, oligonucleotide modification, and purification steps are performed according to the manufacturers' specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references which are provided throughout this document. A basic text disclosing the general methods of use in this invention is Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Publish., Cold Spring Harbor, NY 2nd ed. (1989), which is incorporated herein by reference.

A first step in the synthesis of retroviral vectors of the invention is obtaining an infectious GaLV DNA clone. Proviral DNA from at least three GaLV strains (GaLV SF, GaLV SEATO, and SSAV) has been cloned. A GaLV SF clone including both ends of the GaLV SF genome and the envelope gene but not an intact region of the genome encoding the core proteins is reported by Scott et al. *Proc. Natl. Acad. Sci. USA* 78:4213-4217 (1981). A partial clone containing the envelope and part of the genome but not the region encoding core proteins of SSAV is described by Gelman et al. *Proc. Natl. Acad. Sci. USA* 78:3373-3377(1981). Finally, Gelman et al. *J. Virol.* 44:269-275 (1982) disclose a partial clone of a third GaLV strain, SEATO, containing all but 350 bases of the core region of GaLV. This clone has been sequenced in its entirety by Delassus et al. *Virol.* 173:205-213 (1989) (see Figure 1). The deleted 350 bases were also sequenced but from a PCR fragment

generated from viral RNA expressed in a GaLV SF infected cell line. The sequence of an integrated form of a GaLV SEATO genome is also shown in Seq ID No. 1. All of the above references are incorporated herein by reference.

Example 1 describes the construction of an infectious GaLV clone comprising sequences from GaLV SEATO and GaLV SF. This construction can be used to prepare a number of retroviral vectors, as described in detail below.

Packaging Cells

Packaging cells for use in the present invention may be made from any animal cell, such as CHO cells, NIH 3T3, mink lung cells, D17 canine cells, and MDBK cells. One or both of the core and envelope components can be encoded by GaLV genes. The core and envelope components, however, need not be derived from the same GaLV strain. Indeed, in some embodiments, the core components may be derived from a different species (e.g. MoMLV). For example, the PG13 murine packaging cell line produces virion particles having MoMLV core and GaLV envelope particles (see Miller, *et al.* (1991) *J. Virol.* 65:2220-2224).

To prepare a packaging cell line, an infectious clone of a desired retrovirus (e.g., GaLV SEATO) in which the packaging site (ψ) has been deleted is constructed. Cells comprising this construct will express all GaLV structural proteins but the introduced DNA will be incapable of being packaged. Alternatively, packaging cell lines can be produced by transforming a cell line with one or more expression plasmids encoding the appropriate core and envelope proteins. In these cells, the *gag*, *pol*, and *env* genes can be derived from the same or different retroviruses.

Although certain cells may express the receptor for a retroviral vector, the cells may not be efficiently infected because of a loss of optimum fit between the receptor and the envelope proteins. For example, altered glycosylation patterns may inhibit retroviral infection (Wilson et al., *J. Virol.* 65:5975-5982 (1991), which is incorporated herein by reference). In addition, retroviruses in the same receptor class can exhibit different host ranges due to single amino acid differences in target cell receptors.

In light of these considerations, it may be necessary to modify the envelope proteins of the hybrid virions to adjust the host range. The proteins may be modified to either allow infection of cells previously resistant to infection or to prevent infection of non-target cells.

One strategy for modifying envelope proteins is the use of an *in vitro* selection scheme. In this approach, an infectious clone of the retrovirus along with a selectable marker gene is introduced into target cells that are resistant to infection. Those cells which have been infected by retroviruses comprising mutations allowing infection of the cells are then identified by standard reverse transcriptase assays of the culture supernatant. The *env* gene of the adapted retrovirus is cloned and sequenced and used to construct new retroviral vectors capable of efficiently infecting the target cell. This strategy is particularly useful in isolating variants capable of infecting a number of human cells currently resistant to GaLV infection such as tumor infiltrating lymphocytes, bone marrow cells, stem cells, and hepatocytes.

Alternatively, if the gene encoding the cell receptor has been cloned, the gene can be inserted in a cell line which does not normally produce the receptor. Variant retroviruses capable of binding the receptor can then be identified in the same manner as described above. For instance, the human GaLV cell surface receptor has been cloned and sequenced. U.S. Patent No. 5,151,361, and Johann et al., *J. Virol.* 66:1635-1640 (1992), which are incorporated herein by reference. Thus, this gene can be used to identify new retroviral vectors expressing modified envelope proteins.

A third alternative to modifying the host range of a retrovirus vector is by directly modifying the envelope proteins. Modifications of the sequences encoding the polypeptides may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see*, e.g., Gillman and Smith, *Gene* 8:81-97, (1979) and Roberts, S. et al., *Nature* 328:731-734, (1987), which are incorporated

herein by reference). The effect of the modifications are evaluated by screening for the ability of the engineered virions to infect a target cell.

In addition, specific polynucleotide sequences encoding desired polypeptides can be fused to the *env* gene using methods known to those skilled in the art. Gene fusions comprising sequences encoding antibodies, SCF, IL-6 somatostatin and the like can thus be used as a targeting means. The fused gene can be inserted into an appropriate plasmid for transformation into the packaging cells.

In addition, the envelope protein can be modified for example, by introducing point mutations in the protein to yield moieties for coupling by organic chemical means (e.g., insertion of a cysteine residue to give a sulfhydryl group). Cell-specific targeting moieties can be coupled with glutaraldehyde, periodate, or maleimide compounds, or by other means known to those skilled in the art. Such couplings may also be made directly to wild-type or unmodified envelope proteins where coupling can be to a carbohydrate moiety, a sulfhydryl group, an amino group, or other group which may be available for binding.

A number of packaging cell lines suitable for the present invention are also available in the prior art. These lines include Crip and GPE-Am. Preferred existing cell lines include PA317 (ATCC CRL 9078) which expresses MoMLV core and envelope proteins and PG13 (ATCC CRL 10,683) which produces virions having MoMLV core and GaLV envelope components. (See Miller et al. *J. Virol.* 65:2220-2224 (1991), which is incorporated herein by reference.) The PG13 packging cell line can be used in conjunction with the 521 plasmid and the 537 plasmid, both of which contain 5' MoMLV LTR and packaging signal sequences (see Example 3, herein).

Defective Genomes

The other component of retroviral vectors is a packagable defective genome comprising a polynucleotide sequence, typically a structural gene, of interest. The defective genomes of the invention include a GaLV component which include minimal GaLV nucleotide sequences must be

present in the defective genome itself for the genome to integrate in the target cell genome and be packaged in infectious virions (i.e. the sequences are required in *cis*). Thus, the GalV component of the defective genomes of the invention may include the packaging site, ψ, and/or the long terminal repeated sequences (LTRs). The LTRs are positioned at either end of the proviral DNA and contain regulatory sequences (e.g., promoters, enhancers and polyadenylation sequences) which direct expression of the genes within the proviral DNA. The polynucleotide sequences of the GaLV component may be identical to sequences as shown, for instance, in SEQ ID. No 1, or may be substantially identical to that sequence as defined, above.

Typically, the proviral regulatory sequences drive expression of the inserted gene. In those embodiments where two inserted genes are included (e.g., a marker gene and the gene of interest) it is frequently desirable to include a virus internal ribosome entry site (IRES) to increase efficiency of expression (Ghattas et al., *Mol. Cell. Biol.* 11:5848-5859 (1991), which is incorporated herein by reference).

The promoter operably linked to the gene of interest may be constitutive, cell type-specific, stage-specific, and/or modulatable (e.g., by hormones such as glucocorticoids). Suitable promoters for the invention include those derived from genes such as early SV40, CMV major late, adenovirus immediate early, histone H4, β-actin, MMTV, and HSV-TIC.

Enhancers increase the rate of transcription from promoters, act on *cis*-linked promoters at great distances, are orientation independent, and can be located both upstream, (5'), and downstream, (3'), from the transcription unit. Enhancers inducible by hormones and metal ions and found only in specific tissues have been described. Proteins synthesized only in one tissue type, for example, actin and myosin in muscle, are frequently regulated by tissue specific enhancers. For tissue specific expression of the introduced genes of interest used in the retroviral vectors of the present

invention, tissue-specific enhancers are of particular interest.

A repetitive 45 base pair enhancer element in the U3 region of the GaLV LTR is important for tissue specific expression of the introduced genes. This enhancer region is present only once in the 3' LTR of GaLV SF but is present 3 times in the 3' LTR of GaLV SEATO. (See Quinn *et al., Mol. Cell. Biol.* 7:2735-2744, which is incorporated herein by reference). The sequence of the 3' LTR of GaLV SEATO with 3 repeats of the 45 bp enhancer region is shown in Seq. ID No.2. Thus, the origin of the 3' GaLV LTR region (from GaLV SEATO or GaLV SF) in a retroviral vector can influence the expression of the introduced gene in different tissues (see Example 4, herein).

To ensure efficient expression, 3' polyadenylation regions must be present to provide for proper maturation of the mRNA transcripts. The native 3'-untranslated region of the gene of interest is preferably used, but the polyadenylation signal from, for example, SV40, particularly including a splice site, which provides for more efficient expression, could also be used. Alternatively, the 3'-untranslated region derived from a gene highly expressed in a particular cell type could be fused with the gene of interest.

The retroviral vectors of the invention also contain GaLV-based regulatory elements that can direct expression of genes contained within the genome in a tissue/cell specific manner. In general, the GaLV regulatory elements are more efficient than the MoMLV elements in expressing genes in human cells. In addition, the regulatory sequences from different GaLV strains have different cell and tissue specificities. For instance, GaLV SF regulatory genes function efficiently in primate lymphoid cells (e.g., UCD 144) and GaLV SEATO regulatory genes function efficiently in human myeloid cells (e.g., HL60 cells), while MoMLV regulatory genes do not. Thus, tissue specificity of the vectors of the invention can be modified by selecting the appropriate GaLV strain. Tissue specificity of the regulatory genes from various GaLV strains

is determined using routine screening techniques well-known to those of skill in the art.

The 5' and 3' LTRs of one retrovirus or GaLV strain may be also used in a defective genome derived from another. For instance, the 3' LTR from SSAV can be substituted for the 3' LTR of an infectious clone of another GaLV strain. Since the U3 region of the 3' LTR is the template for the synthesis of the U3 region in both 5' and 3' LTRs of the progeny virus, the 3' LTR will be duplicated and transferred to the 5' LTR in the host cell. In this way optimal expression of the gene of interest in the target cell can be achieved.

In addition, in order to increase efficiency of packaging, the 5'LTR from one virus (e.g., MoMLV) can be used in combination with the 3' LTR of a second (e.g., GaLV). If the constructs comprise a MoMLV 5'LTR and a GaLV 3'LTR, they are efficiently expressed in murine packaging cells (e.g., PG13) but result in proviral DNA comprising promoter sequences from GaLV which function more efficiently in human cells. These constructs are efficiently packaged in packaging cells such as PG13 because the 5' MoMLV LTR drives gene transcription in the packaging cells. However, when the packaged retrovial vector is infected into an appropriate target cell, the 3' GaLV promoter drives gene transcription (see Example 3, herein). Examples of retroviral vectors with MoMLV 5' LTR's and packaging signals and 3'GaLV LTR's include plasmids 521 and 537, described in Example 3, herein. This type of retroviral vector has the advantages of both efficient packaging in cell lines such as PG13 and higher expression in various target cells (see Example 4, herein).

The cis-acting packaging sequences used in the defective viral genomes may be derived from GaLV SEATO. The minimal sequences required for efficient packaging of a GaLV-based defective genome are described herein. In particular, as shown in detail below, the first 910 to 1290 nucleotides from the 5' end of the GaLV SEATO genome can direct packaging of a defective genome by PG13 and PA317 cells. This result also shows that the sequences required for efficient packaging from GaLV are recognized by MoMLV core proteins. Thus, hybrid

retroviral vectors comprising both GaLV and MoMLV components can be conveniently constructed.

The GaLV SEATO sequences required for packaging of the defective genomes include the 5' LTR and extend to about position 1290 of the GaLV genome illustrated in Figure 1. The sequences required for packaging also include the packaging site, ψ, which is typically defined negatively as a sequence which, when deleted from a viral genome, prevents efficient packaging of the genome. In the GaLV SEATO genome, ψ is located downstream of the 5' LTR beginning at about position 200. The site usually comprises at least about 350 bp, preferably between about 500 bp and about 1500 bp, more preferably about 700 to about 1200 bp. One of skill will recognize that minor modifications to the packaging sequence shown in Figure 1 will not substantially affect the ability of the sequence to direct packaging. Thus, the term "GaLV packaging site" as used herein refers to GaLV DNA sequences, or RNA sequences transcribed from them which are capable of directing packaging when present in *cis* in a GaLV genome or defective genome. The term "GaLV SEATO packaging sites" refers to those DNA or RNA sequences substantially identical (as determined above) to the disclosed sequences and which are functional in the defective GALV genomes of the present invention.

The retroviral vectors of the invention are suitable for delivering a variety of polynucleotides to cells, including transgenes for augmenting or replacing endogenous genes in gene therapy or for the production of transgenic animals. Antisense polynucleotides can be used to control expression of target endogenous genes such as oncogenes. In addition, genes encoding toxins can be targeted for delivery to cancer cells. Other suitable sequences include those encoding growth substances to promote immune responses to cancers or infections, soluble factors to modulate receptor activity, and the like. The inserted polynucleotide of interest should be less than about 10 kb, preferably between about 7 and 8 kb.

In certain embodiments, homologous targeting constructs are used to replace an endogenous target gene. Methods and materials for preparing such constructs are known by those of skill in the art and are described in various references. See, e.g., Thomas et al., *Cell* 51:503 (1987) and Capecchi, *Science* 244:1288 (1989), which are incorporated herein by reference.

Homologous targeting constructs have at least one region having a sequence that substantially corresponds to, or is substantially complementary to, a predetermined endogenous target gene sequence (e.g., an exon sequence, an enhancer, a promoter, an intronic sequence, or a flanking sequence of the target gene). Such a homology region serves as a template for homologous pairing and recombination with substantially identical endogenous gene sequence(s). In the targeting of transgenes, such homology regions typically flank the replacement region, which is a region of the targeting transgene that is to undergo replacement with the targeted endogenous gene sequence. Thus, a segment of the targeting transgene flanked by homology regions can replace a segment of the endogenous gene sequence by double crossover homologous recombination.

In addition, the constructs for both homologous targeting and random integration will comprise a selectable marker gene to allow selection of cells. Frequently, multiple selectable marker genes are incorporated, such as in positive-negative selection constructs for homologous gene targeting.

A selectable marker gene expression cassette typically comprises a promoter which is operational in the targeted host cell linked to a structural sequence that encodes a protein that confers a selectable phenotype on the targeted host cell, and a polyadenylation signal. A promoter included in an expression cassette may be constitutive, cell type-specific, stage-specific, and/or modulatable (e.g., by hormones such as glucocorticoids; MMTV promoter), but is expressed prior to and/or during selection.

When the selectable marker is contained in a homologous targeting construct, homologous recombination at

the targeted endogenous site(s) can be chosen to place the selectable marker structural sequence downstream of a functional endogenous promoter, and it may be possible for the targeting construct replacement region to comprise only a structural sequence encoding the selectable marker, and rely upon an endogenous promoter to drive transcription. Similarly, an endogenous enhancer located near a targeted endogenous site may be relied on to enhance transcription of selectable marker gene sequences in enhancerless constructs.

Suitable selectable marker genes include, for example: *gpt* (encoding xanthine-guanine phosphoribosyltransferase), which can be selected for with mycophenolic acid; *neo* (encoding neomycin phosphotransferase), which can be selected for with G418, and DFHR (encoding dihydrofolate reductase), which can be selected for with methotrexate. Other suitable selectable markers will be apparent to those in the art.

Selection for correctly targeted recombinant cells will generally employ at least positive selection, wherein a selectable marker gene expression cassette encodes and expresses a functional protein (e.g., *neo* or *gpt*) that confers a selectable phenotype to targeted cells harboring the endogenously integrated expression cassette, so that, by addition of a selection agent (e.g., G418, puromycin, or mycophenolic acid) such targeted cells have a growth or survival advantage over cells which do not have an integrated expression cassette.

Cells harboring the transgene of interest either randomly integrated or integrated by homologous recombination may be further identified using techniques well known in the art. For instance, the cells can be screened using Southern blotting or the polymerase chain reaction (PCR). If targeted integration is being screened, the oligonucleotide probes or PCR primers should bracket recombination junctions that are formed upon transgene integration at the desired homologous site.

Gene Therapy

The retroviral vectors of the invention are particularly suitable for delivering polynucleotides to cells for gene therapy of a number of diseases. Current strategies for gene therapy are reviewed in Friedmann, *Science* 244:1275 (1989), which is incorporated herein by reference.

Delivery of the polynucleotide of interest may be accomplished *in vivo* by administration of the vectors to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion). Alternatively, the vectors may be used to deliver polynucleotides to cells *ex vivo* such as cells explanted from an individual patient (e.g., tumor-infiltrating lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the polynucleotide.

The vectors may be used for gene therapy to treat congenital genetic diseases, acquired genetic diseases (e.g., cancer), viral diseases (e.g., AIDS, mononucleosis, herpesvirus infection, cytomegalovirus infection, papillomovirus infection) or to modify the genome of selected types of cells of a patient for any therapeutic benefit. Treatable disorders include hemophilia, thalassmias, ADA deficiency, familial hypercholesterolemia, inherited emphysema, cystic fibrosis, Duchenne's muscular dystrophy, lysosomal storage diseases, Gaucher's disease, and chronic granulomatous disease.

The vectors of the invention can be used to introduce polynucleotides into a variety of cells and tissues including myeloid cells, bone marrow cells, lymphocytes, hepatocytes, fibroblasts, lung cells, and muscle cells. For example, polynucleotides conferring resistance to a chemotherapeutic agent may be transferred to non-neoplastic cells, especially hematopoietic cells. Alternatively, polynucleotides comprising a toxin gene (e.g., ricin or diphtheria toxin) expression cassette or a negative selectable marker gene expression cassette may be selectively inserted

into neoplastic cells. Expression of the toxin gene or negative selection gene (followed by negative selection) selectively kills target cells. Polynucleotides which are not cytotoxic but which reverse or suppress the neoplastic phenotype (e.g. antisense inhibition of oncogene expression) also may be used to treat cancer, as well. Other uses include the introduction of immunomodifiers into bone marrow cells to treat cancers.

Transgenic Animals

As noted above, the vectors of the present invention are particularly useful for gene targeting mediated by homologous recombination between a targeting polynucleotide construct and a homologous chromosomal sequence. In addition to gene therapy, such strategies are also useful for the production of transgenic animals.

The ability to introduce new genes into the germ line of an animal has been extremely valuable for basic understanding of gene expression. The improvement of desired traits in agricultural or domesticated animals is also possible using these techniques. For example, potential new traits that may be introduced include sterility in meat producing strains of cattle, or fertility and milk production in dairy cows. Other commercially desirable properties include hardiness and rapid weight gain in livestock, or "show qualities" in domestic animals such as dogs and cats. For a review of the genetic engineering of livestock see, Pursel et al, *Science* 244:1281 (1989), which is incorporated herein by reference.

Typically, embryonic stem (ES) cells are used as the transgene recipients. Cells containing the newly engineered gene are injected into a host blastocyst, which is reimplanted into a recipient female. Some of these embryos develop into chimeric animals that possess germ cells partially derived from the mutant cell line. By breeding the chimeric animals it is possible to obtain a new line containing the introduced gene.

The following examples are provided by way of illustration, not limitation.

Example 1

Construction of GaLV infectious clone comprising GaLV SEATO and GaLV SF sequences.

To prepare the GaLV infectious clone, a missing fragment of about 250 kb from the *pol* gene of a GaLV SEATO clone was replaced with the corresponding sequence from GaLV SF. The following steps correspond to the numbered steps illustrated in Figures 2A-2F.

The steps illustrated in Figure 2A show repair of *pol* gene of GaLV-SEATO.

1 The approximately 8.5 kb permuted GaLV-SEATO provirus (pGAS-2 Hd1) from pGAS-2 (Gelman et al., 1982, *supra*) was isolated by HindIII digestion and DEAE-cellulose membrane interception in an agarose gel. An approximately 250 bp GaLV-SF *pol* gene fragment of pGV-3 corresponding to the missing *pol* fragment of PGAS-2 was isolated by HindIII digestion and DEAE-cellulose membrane interception in an agarose gel.

2 The two DNA species were ligated at low concentration to favor circularization over multimer formation.

3 After ligated material was precipitated, Sal I restriction was used to linearize the construct.

4 The construct was ligated into Sal I-restricted and phosphatased pVZ-1 vector.

5 DH5αF' cells were transformed.

6. Transformants were screened by alkaline lysis, plasmid mini-preps, and sequencing with "GVGAS 10" primer to check number and orientation of GaLV-SF *pol* fragment inserts within GaLV-SEATO sequence. A clone with correct construction was named intermediate Clone 66.

Figure 2B shows change of GaLV-SEATO insert orientation.

7 The permuted proviral Clone 66 insert was isolated by Sal I digestion and DEAE-cellulose membrane interception on an agarose gel.

8 The insert was re-ligated back into pVZ-1 Sal I-cut and phosphatased vector to obtain opposite orientation. The opposite orientation clone was named intermediate Clone 120.

Figures 2C and 2D illustrate the intermediate Clone 66 and the unidirectional decrease in insert length using Exonucleases III and VII.

9 Intra-insert distances were estimated by known sequence and accurate restriction mapping. The goal was to decrease the 8.5 kb insert by 5.4 kb, stopping at a point just 3' of the LTR-LTR junction, leaving one LTR intact. The size of resulting clone (vector + insert) was ~ 6 kb.

10 Not I restriction of Clone 66 and Clone 120 was used to check for absence of intra-insert sites. They were found to be absent. Clone 66 was linearized with Not I in the multiple cloning site.

11 The Not I termini were filled in with cold dCTP[αS] and dGTP[αS] and DNA polymerase I (Klenow). α-thiodeoxyribonucleotides were used to block these termini from Exonuclease III digestion.

12 Clone 66 and Clone 120 were restricted with Xba I to check for absence of intra-insert sites. Clone 66 was restricted with Xba I in the multiple cloning site generating 5' overhang cohesive termini.

13 Precisely timed Exonuclease III digestion destroyed the Xba I site but the Sal I site at 5' insert end was left intact, and incomplete Not I site was resistant to attack by Exonuclease III.

14 Digestion with Exonuclease VII was used to remove remaining single strand.

15 The "ragged ends" were filled in with DNA polymerase (Klenow) and cold deoxynucleotide triphosphates.

16 The blunt ended incomplete Not I site was ligated to insert sequence.

17 DH5αF' cells were transformed.

18 Transformants were screened by alkaline lysis, plasmid mini-preps, Sal I linearization and sequencing to determine (a) extent of insert deletion and (b) quality of incomplete Not I sites and the true extent of protection given by α-thiodeoxyribonucleotides from digestion into the vector by Exonuclease III or VII.

19 Transformants were further screened by Not I digestion, searching for complete Not I site.

20 Clones that linearize with Not I were linearized to confirm presence of complete Not I site and accurately determine extent of insert deletion. One clone with desired digestion to a point just 3' of the LTR-LTR junction and with a complete Not I site, was named intermediate Clone 66Exo52.

Figure 2E shows the intermediate Clone 120: Unidirectional decrease in insert length using Exonucleases III and VII.

21 Intra-insert distance was estimated by known sequence and accurate restriction mapping. The goal was to decrease the 8.5 kb insert by 2.6 kb, stopping at a point just 3' of the LTR-LTR junction leaving one LTR intact. Size of resulting clone was ~ 9 kb.

22 to 32 The steps were preformed as described for steps 10-20. One clone with desired digestion to a point just 3' of the LTR-LTR junction and with a complete Not I site, was named Intermediate Clone 120Exo55.

Figure 2F shows coupling of Clone 66Exo52 insert and Clone 120Exo55 insert: separation of LTR's and generation of infectious clone.

33 Double digestion of both Clone 66Exo52 and Clone 120Exo55 with Sal I and Not I was used to release inserts.

34 Inserts were isolated by DEAE cellulose membrane interception in agarose gels.

35 Ligation of Clone 66Exo52 insert, Clone 120Exo55 insert and Not I restricted pVZ-vector.

36 DH5αF' cells were transformed.

37 Screening of transformants by ^{32}P-labelled probing of colonies, alkaline lysis plasmid mini-preps, restriction analysis and sequencing to search for potential infectious clones with correct construction.

38 Large scale plasmid preparation and restriction mapping of GaLV-SEATO infectious clone.

The resulting cloned GaLV genome was subsequently shown to encode infectious GaLV virions.

Example 2

Construction of defective genomes comprising GaLV SF and GaLV SEATO packaging sites.

The steps used to prepare a defective genome comprising GaLV SEATO sequences from the infectious clone in Example 1 were as follows.

1. A 1667 bp Not I-Bgl II fragment from the 5' end of the infectious clone of GaLV SEATO was isolated.

2. A 3116 bp Bam HI-Xba I fragment corresponding to the Lac Z gene was isolated from the p1203 Lac Z plasmid (Ghattas et al., *supra*).

3. A 596 bp Xba I to Hind III fragment corresponding to the ECMV IRES (EMCV internal ribosome entry site) was isolated from pLZIC2 (Ghattas et al., *supra*).
4. A 890 bp Stu I- Sfu I fragment corresponding to the G418 resistance gene was isolated from pRcCMV plasmid (Invitrogen).
5. A 995 bp Stu I-Not I fragment corresponding to the 3' end of the GaLV SEATO infectious clone was isolated.
6. A linearized Not I pGem 13 plasmid (Promega, 3181bp) was isolated.
7. These fragments were ligated together to assemble the pGaLV SEATO 395 plasmid.

Figure 3 (top) shows the resulting defective genome. Figure 3 (middle) shows a defective genome constructed in the same manner but using a Not I-Nco I fragment from the 5' end of the GaLV SEATO genome. Figure 3 (bottom) shows a construct prepared from GaLV SF sequences.

The pGaLV SEATO 395 plasmid was further modified by increasing the length of the 5' putative packaging region by 328 bp in creating the GaLV SEATO 558 construct. Plasmid 558 this represents a modified 395 plasmid which contains an additional 328 nucleotides of 5' GaLV SEATO sequences extending to the Bgl II site at position 1290 of the GaLV genome. (Plasmid 395 extends only to the Nco I site at position 910 of the GaLV genome.) The 558 plasmid construction was made using the 194 GaLV SF plasmid. The GaLV SF 194 plasmid contains a truncated GaLV SF genome cloned into the Promega pSP72 genome at the Eco RI site.

The steps in construction of the 558 plasmid are listed below.

1. A Pst I- Bgl II fragment of GaLV SEATO containing the 5' GaLV SEATO LTR and the GaLV SEATO packaging site was used to replace the corresponding region of the GaLV SF 194 plasmid partial genome.
2. A Barn HI-Xba I fragment containing the bacterial Lac Z gene but lacking an initiation codon was ligated, in reading frame, to the Bgl II site such that the Lac Z

gene initiated from the GaLV SEATO gag protein translation start codon. Therefore the β-galactosidase protein is a GaLV SEATO gag-Lac Z fusion protein.

3. An Xba Ito Nsi I fragment containing the EMCV IRES and a G418 gene was ligated to the Xba I site downstream of the Lac Z gene and the Nsi I in the 3' region of the GaLV SF 194 genome.

4. The Nsi I- Sma I region at the 3' end of the 194 GaLV SF genome was replaced with a corresponding region of GaLV SEATO, such that the 3' U3 of the LTR contained GaLV SEATO derived sequences in place of the GaLV SF 194 sequences.

The schematic diagrams of plasmids 395 and 558 are compared in figure 4 and the nucleotide sequence of plasmid 558 is shown in Seq. ID No. 3.

Example 3

Construction of GALV defective genomes with improved packaging efficiency in murine packaging cell lines that express MoMLV structural proteins

In order to improve the efficiency of packaging in murine packaging cell lines such as PG13 and PA317, which express MoMLV structural proteins, we constructed GALV defective gemomes that have a MoMLV promoter at the 5' end and a GaLV promoter at the 3' end.

Two defective genomes, designated plasmid 521 and plasmid 537, having a MoMLV promoter at the 5' end and a GaLV promoter at the 3' end, were constructed. In order to construct plasmid 521, the 5' end of the 395 plasmid (Sfi I/filled in-Cla I) was replaced with the corresponding fragment of a similar MoMLV-based Lac Z genome (Sst II/filled in to Cla I). In order to construct plasmid 537, the 3' Nsi I- Not I (filled in) fragment of 521 was replaced with Nsi-Bgl II (filled in) fragment of GaLV SF 194.

For comparative purposes, a MoMLV defective genome plasmid similar in construction to the 521 plasmid, was prepared by replacing the Spe I- Sph I fragment of pLXSN (which contains the end of the MoMLV packaging region, the SV40 promoter and the 5' part of the G418 gene with the

corresponding region (also an Spe I-Sph fragment) of the 521 genome, thereby replacing the SV40 promoter with an IRES element. This defective genome is designated plasmid 560. Plasmids 521, 537, and 560 are shown schematically in figures 4 and 5. The nucleotide sequence of plasmid 521 is shown in Seq. ID No. 4 and the nucleotide sequence of plasmid 537 is shown in Seq. ID No. 5.

The 521 and 537 plasmid constructs provide a means of optimizing gene expression in the packaging cells while retaining GaLV-driven gene expression in target cells where GaLV promoters function more efficiently then the MoMLV promoter. Because the 521 and 537 constructs have a MoMLV promoter at the 5' end, cells *transfected* with these constructs (such as packaging cells PA317 and PG 13) have a MoMLV promoter (U3) driving gene transcription. On the other hand, when the genome is reverse transcribed after infection of the target cell, the GaLV U3 promoter in the 3' LTR is duplicated and replaces the MoMLV promoter at the 5' end. This has been demonstrated by sequence analysis of unintegrated vector DNA from 521 target cells (data not shown). The DNA from these cells infected with the 521 construct after packaging in either PG13 or PA317 cells contains a 5' AND 3' GaLV SEATO U3 (data not shown). Therefore the 5' end of the 521 genome switches from a MoMLv U3 to a GaLV SEATO U3 in infected cells, which results in GaLV-driven gene expression in target cells.

Example 4

Effect of the number of 45 bp enhancer elements in the U3 region of the GaLV LTR on efficiency of gene expression in target cells

There are a variable number of repetitive 45 bp enhancer elements in the U3 region of the GaLV LTR. The 558 plasmid and the 521 plasmid U3 regions, derived from GaLV SEATO, each contain 3 repetitive 45 bp enhancer elements, whereas GaLV SF (eg. plasmids 537 and 559) has only one of these elements. The number of repeats may play a restrictive but potentially useful role in governing expression of downstream genes in different target cells. The experimental

data presented below suggests that the number of repetitive 45 bp enhancer elements in the U3 region of the LTR of GaLV can effect the efficiency of tissue/cell specific gene expression.

Following transfection of the 521, 537 or 560 plasmids into the PA317 or PGI3 cell lines, the MoMLV promoters are used to express packagable genomes. For the 521 and 537 plasmids, however, the GaLV promoter is used to express β-galactosidase and G418 resistance in the target cell after infection with the packaged defective genomes. The effect of three repeats of the 45 basepair enhancer region versus only one copy of the enhancer region in the GaLV promoter is shown in the table below. The expression of the G418 indicator gene is measured by titering G418 resistant colonies. The data in the table below demonstrates the effect of varying the number of 45 bp enhancer region repeats on the expression of genes driven by the GaLV LTRs in different cell types (see table).

Table: Efficiency of Gene Expression Directed by Retroviral Vectors in Various Target Cells

genome	537	558	560
packaging cells:	PGI3	PGI3	PGI3
promoter used	GaLV SF	GaLV SEATO	MoMLV
target cells:			
mink fibroblasts	$2x10^{2\#}$	$5x10^4$	5.0
murine NIH 3T3 cells	$5x10^4$	5.0	$5x10^4$
BHK hamster cells	-	0.5x10	-
HaK hamster cells	-	$0.5x10^2$	-
Bovine MDBK cells	$5x10^3$	$5x10^4$	$5x10^2$
	-*	-*	-*
Human KB cells	$5x10^4$	$5x10^2$	5x10
Human HeLA cells	$5x10^4$	$5x10^2$	$5x10^2$
Human 293 cells	5x10	$5x10^2$	$5x10^4$
	$5x10^{3*}$	5x10*	$5x10^{3*}$

\# titer expressed as number of G418 resistant colonies obtained with I ml of PGI3 or PA317 supernatant

containing retroviral vectors with either the 537, 558 or 560 genomes

* genomes packaged in PA317 cells

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(I) TELEX:

(ii) TITLE OF INVENTION: Gibbon Ape Leukemia Virus-Based Retroviral Vectors

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO not yet assigned
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(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..8535
(D) OTHER INFORMATION: /standard_name= "GaLV SEATO Genome"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATGAAAGAA GTGTTTTTTT TTAGCCAACT GCAGTAACGC CATTTTGCTA GGCACACCTA 60

AAGGATAGGA AAAATACAGC TAAGAACAGG GCCAAACAGG ATATCTGTGG TCATGCACCT 120

GGGCCCCGGC CCAGGCCAAG GACAGAGGGT TCCCAGAAAT AGATGAGTCA ACAGCAGTTT 180

CCAGCAAGGA CAGAGGGTTC CCAGAAATAG ATGAGTCAAC AGCAGTTTCC AGGGTGCCCC 240

TCAACCGTTT CAAGGACTCC CATGACCGGG AATTCACCCC TGGCCTTATT TGAACTAACC 300

AATTACCTTG CCTCTCGCTT CTGTACCCGC GCTTTTTGCT ATAAAAATAA GCTCAGAAAC 360

TCCACCCGGA GCGCCAGTCC TTAGAGAGAC TGAGCCGCCC GGGTACCCGT GTGTCCAATA 420

AAACCTCTTG CTGATTGCAT CCGGAGCCGT GGTCTCGTTG TTCCTTGGGA GGGTTTCTCC 480

TAACTATTGA CCGCCCACTT CGGGGGTCTC ACATTTGGGG GCTCGTCCGG GATCGGAAAC 540

CCCACCCAGG GACCACCGAC CCACCAACGG GAGGTAAGCT GGCCAGCGAC CGTTGTGTGT 600

CTCGCTTCTG TGTCTAAGTC CGTAATTCTG ACTGTCCTTG TGTGTCTCGC TTCTGTGTCT 660

GAGACCGTAA CTCTGACTGC CCTTGTAAGT GCGCGCATTT TTTTGGTTTC AGTCTGTTCC 720

GGGTGAATCA CTCTGCGAGT GACGTGTGAG TAGCGAACAG ACGTGTTCGG GGCTCACCGC 780

CTGGTAATCC AGGGAGACGT CCCAGGATCA GGGGAGGACC AGGGACGCCT GGTGGACCCC 840

TCGGTAACGG GTCGTTGTGA CCCGATTTCA TCGCCCGTCT GGTAAGACGC GCTCTGAATC 900

TGATTCTCTC TCTCGGTCGC CTCGCCGCCG TCTCTGGTTT CTTTTTGTTT CGTTTCTGGA 960

AAGCCTCTGT GTCACAGTCT TTCTCTCCCA AATCATCAAT ATGGGACAAG ATAATTCTAC 1020

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CGTGGGGTGG CCACCGGAGG GAACTTTTAA TCTCTCTGTC ATTTTTGCAG TTAAAAAGAT 1200

TGTCTTTCAG GAGAACGGGG GACATCCGGA CCAAGTTCCA TATATCGTGG TATGGCAGGA 1260

CCTCGCCCAG AATCCCCCAC CATGGGTGCC AGCCTCCGCC AAGGTCGCTG TTGTCTCTGA 1320

TACCCGAAGA CCAGTTGCGG GGAGGCCATC AGCTCCTCCC CGACCCCCCA TCTACCCGGC 1380

AACAGACGAC TTACTCCTCC TCTCTGAACC CACGCCCCCG CCCTATCCGG CGGCACTGCC 1440

ACCCCCTCTG GCCCCTCAGG CGATCGGACC GCCGTCAGGC CAGATGCCCG ATAGTAGCGA 1500

TCCTGAGGGG CCAGCCGCTG GGACCAGGAG TCGCCGTGCC CGCAGTCCAG CAGACAACTC 1560
GGGTCCTGAC TCCACTGTGA TTTTGCCCCT CCGAGCCATA GGACCCCCGG CCGAGCCCAA 1620
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: LTR
(B) LOCATION: 1..564
(D) OTHER INFORMATION: /standard_name= "3' LTR of GaLV SEATO"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CGCCAGTCCT TAGAGAGACT GAGCCGCCCG GGTACCCGTG TGTCCAATAA AACCTCTTGC 480

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9661 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..9613
(D) OTHER INFORMATION: /standard_name= "p558 retoviral vector"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10306 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..10258
(D) OTHER INFORMATION: /standard_name= "p521 retroviral vector"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10970 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..10970
(D) OTHER INFORMATION: /standard_name= "p537 retroviral vector"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA 10920

GCTATGACCA TGATTACGCC AAGCTATTTA GGTGACACTA TAGAATACTC 10970

WHAT IS CLAIMED IS:

1. A recombinant DNA construct comprising a defective viral genome comprising a polynucleotide sequence of interest and a gibbon ape leukemia virus (GaLV) component.

2. The construct of claim 1, wherein the GaLV component includes a GaLV packaging site.

3. The construct of claim 2, wherein the packaging site consists of between about 150 base pairs and about 1500 base pairs.

4. The construct of claim 2, wherein the packaging site consists essentially of a sequence extending from about position 200 to about position 910 of the sequence shown in Figure 1.

5. The construct of claim 1, wherein the GaLV component includes regulatory sequences which direct expression of the polynucleotide of interest.

6. The construct of claim 5, wherein the regulatory sequences are from a GaLV 3' LTR.

7. The construct of claim 6, wherein the promoter is from GaLV SF.

8. A mammalian cell comprising the defective viral genome of claim 1.

9. The cell of claim 8, further comprising retroviral *gag* and *pol* genes.

10. The cell of claim 9, wherein the *gag* and *pol* genes are from GaLV SF or GaLV SEATO.

11. The cell of claim 9, wherein the *gag* and *pol* genes are from MoMLV.

12. The cell of claim 8, further comprising a retroviral *env* gene.

13. The cell of claim 12, wherein the *env* gene is from GaLV SF or GaLV SEATO.

14. The cell of claim 8, which is PG13 or PA317.

15. An isolated hybrid virion comprising GaLV envelope proteins and an RNA genome comprising a polynucleotide sequence of interest and a GaLV component.

16. The virion of claim 15, further comprising GaLV core proteins.

17. The virion of claim 15, further comprising MoMLV core proteins.

18. The virion of claim 15, wherein the envelope proteins are GaLV SF proteins.

19. The virion of claim 15, wherein the GaLV sequence includes a packaging site.

20. The virion of claim 19, wherein the packaging site is transcribed from a sequence consisting of between about 150 base pairs and about 1500 base pairs.

21. The virion of claim 19, wherein the packaging site is transcribed from a polynucleotide sequence extending from about position 200 to about position 910 of the sequence shown in Figure 1.

22. An isolated recombinant DNA construct comprising a polynucleotide sequence which encodes an

infectious GaLV virion capable of infecting a mammalian cell and producing infectious viral progeny.

23. The construct of claim 22, wherein the DNA construct comprises GaLV SF sequences and GaLV SEATO sequences.

24. The construct of claim 23, wherein the DNA construct comprises 97% GaLV SEATO sequences and 3% GaLV SF sequences.

25. A method of introducing a polynucleotide of interest into human cells having a GaLV receptor, the method comprising:

contacting the cells with hybrid virions comprising GaLV envelope proteins and an RNA genome comprising the polynucleotide sequence of interest and a GaLV packaging site; and

selecting cells having the polynucleotide of interest.

26. The method of claim 25, further comprising implanting the cells in a human patient.

27. The method of claim 25, wherein the human cells are selected from the group consisting of bone marrow cells and tumor infiltrating cells.

GENETIC ORGANIZATION OF GaLV

207

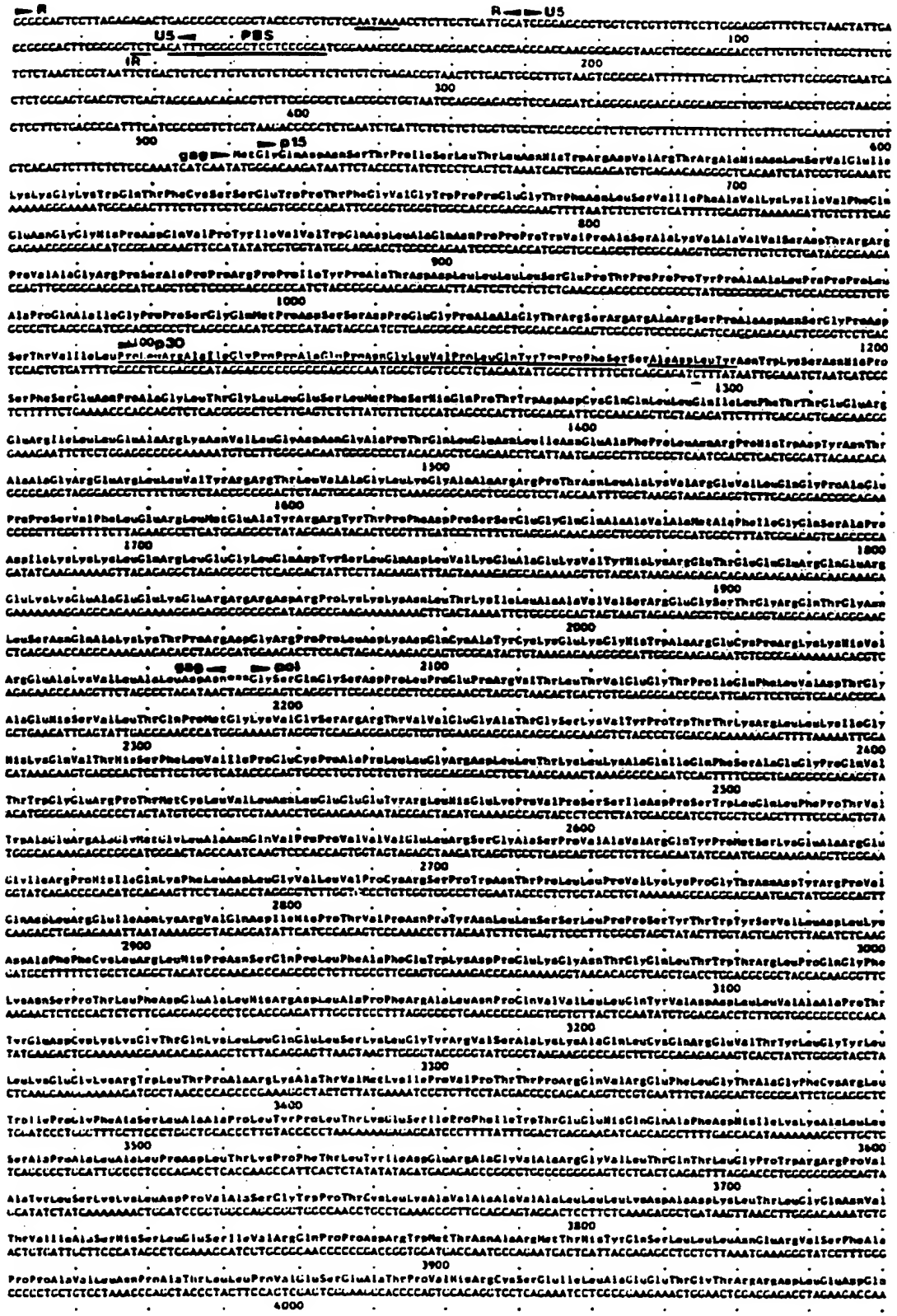


Figure 1

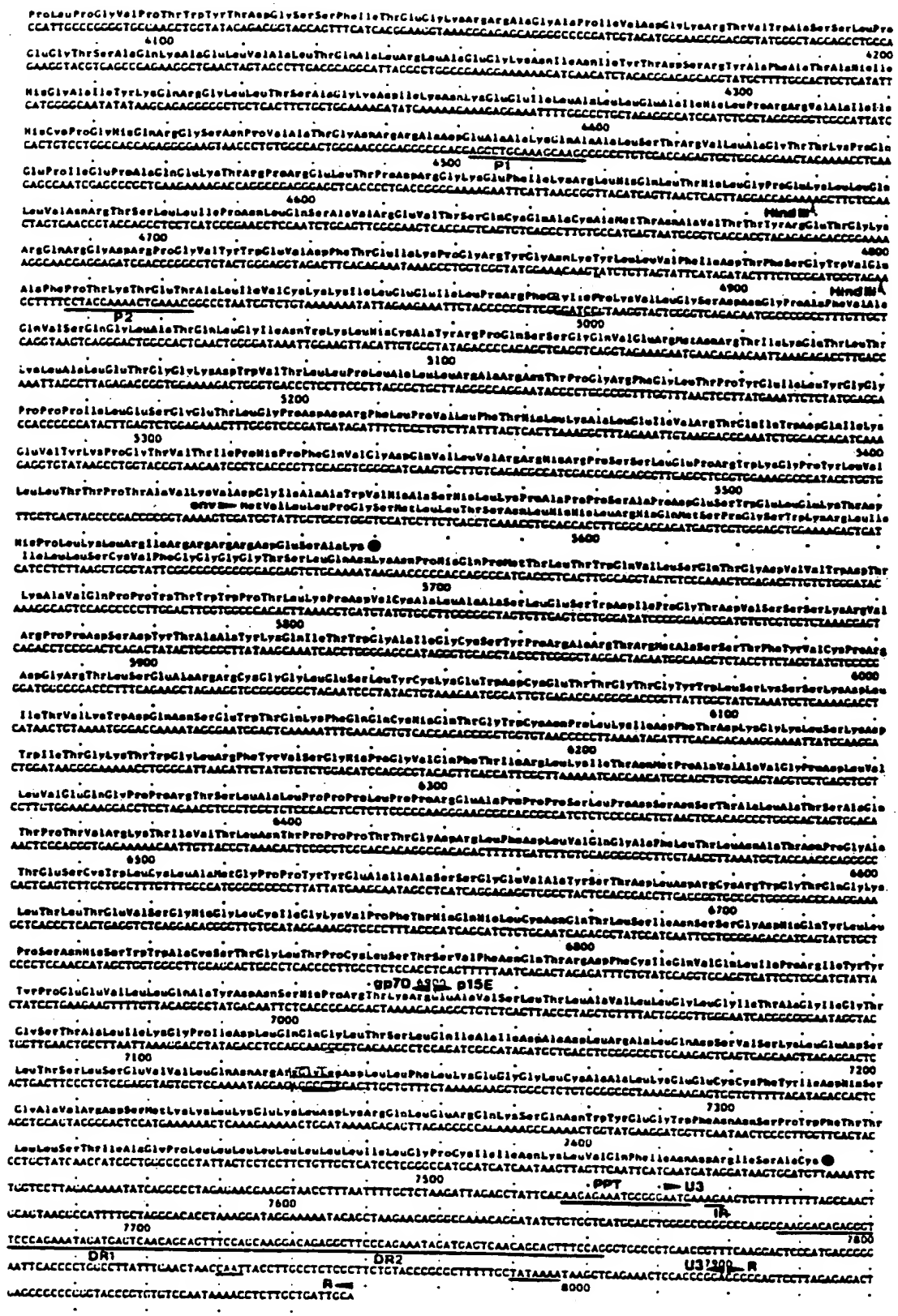


FIG. 1—*Continued*

FIGURE 2A

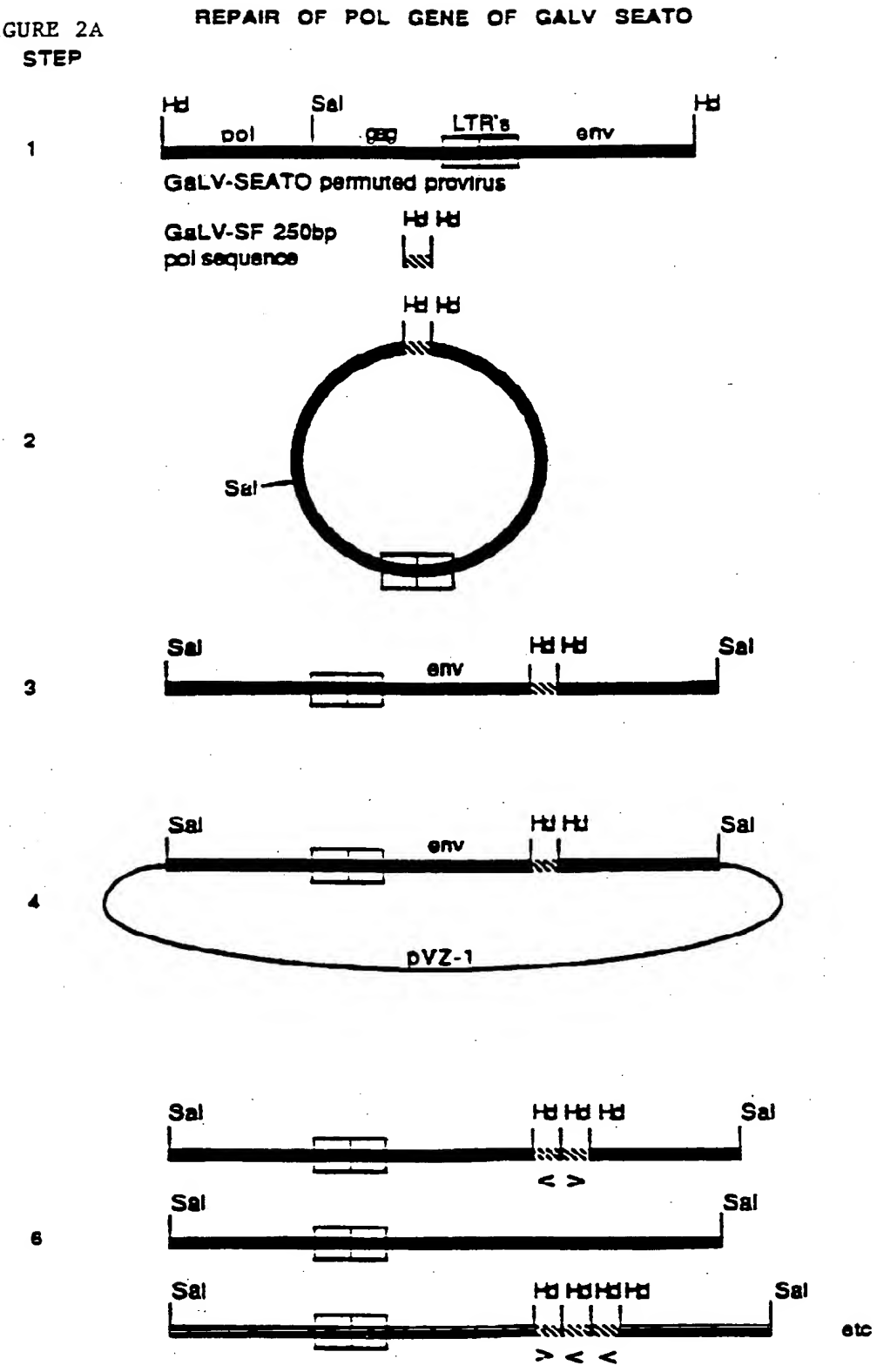
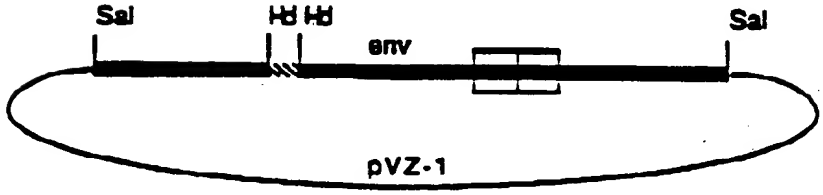

REPAIR OF POL GENE OF GALV SEATO
STEP
1
Hd
Sal
Hd
pol
gag
LTR's
env
GaLV-SEATO permuted provirus
GaLV-SF 250bp
pol sequence
Hd Hd
2
Hd Hd
Sal
3
Sal
env
Hd Hd
Sal
4
Sal
env
Hd Hd
Sal
pVZ-1
5
Sal
Hd Hd Hd
Sal
< >
6
Sal
Sal
Sal
Hd Hd Hd Hd
Sal
> < <
etc

FIGURE 2B CHANGE OF GALV SEATO INSERT ORIENTATION

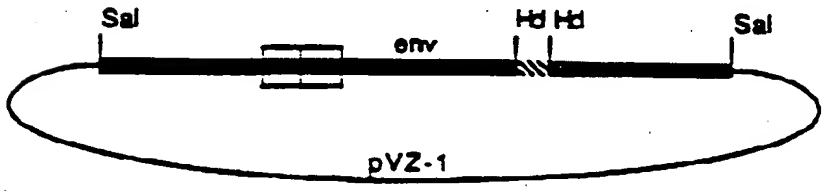
STEP

7

8



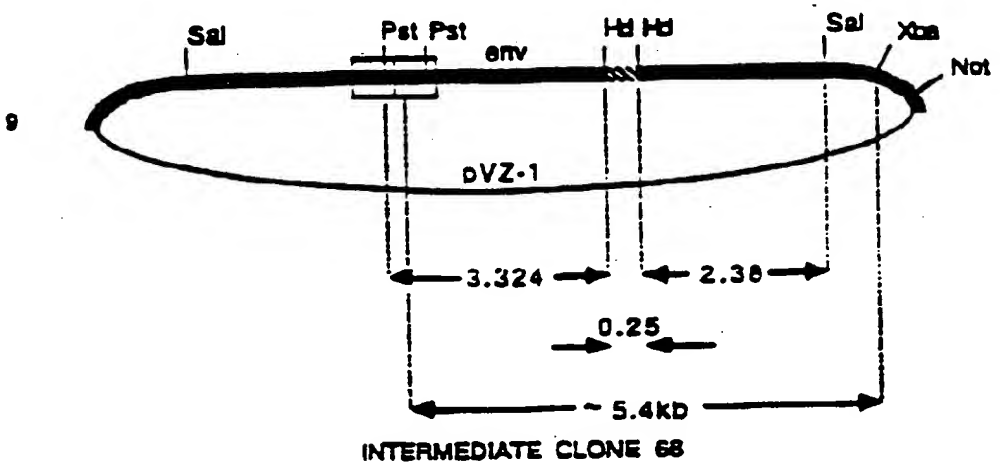
INTERMEDIATE CLONE 120



INTERMEDIATE CLONE GG

FIGURE 2C INTERMEDIATE CLONE 68: UNIDIRECTIONAL DECREASE IN INSERT LENGTH USING EXONUCLEASES III AND VII

STEP



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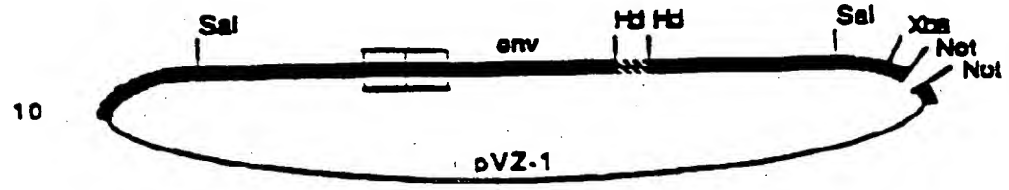

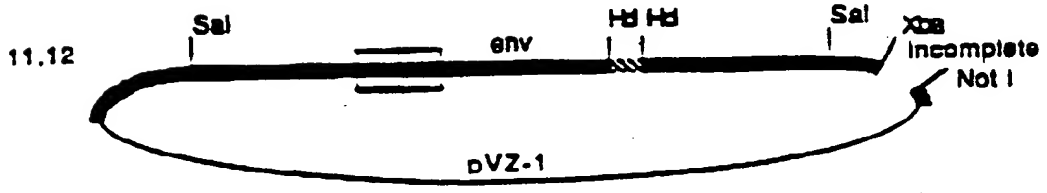

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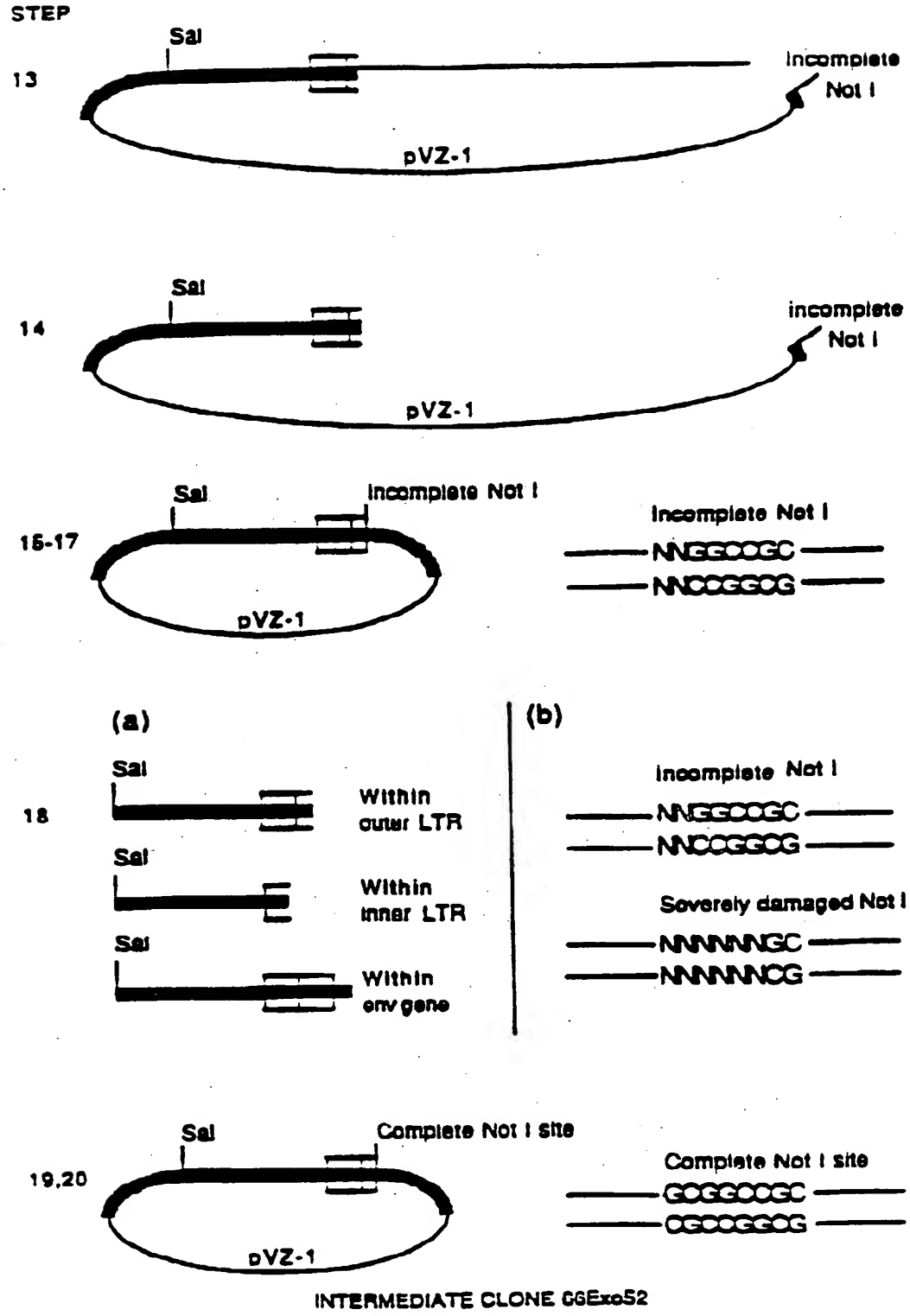
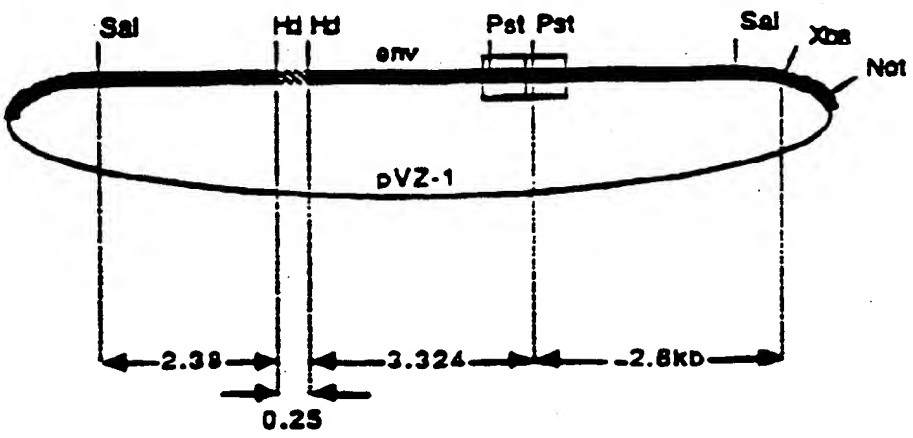
FIGURE 2D



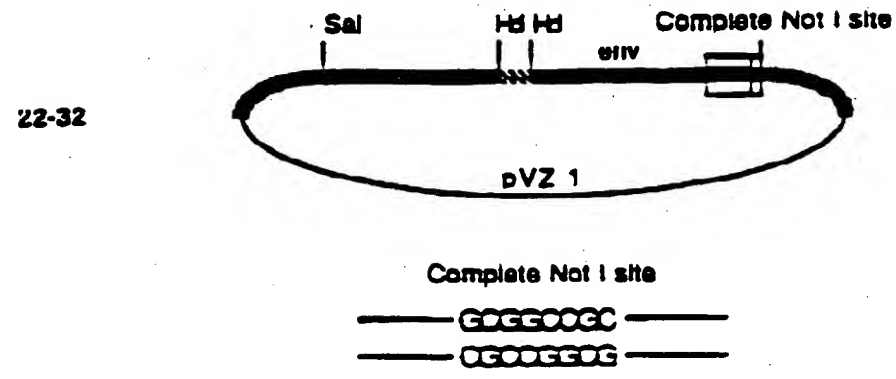
INTERMEDIATE CLONE GGExoS2

FIGURE 2E INTERMEDIATE CLONE 120: UNIDIRECTIONAL DECREASE IN INSERT LENGTH USING EXONUCLEASES III AND VII

STEP



INTERMEDIATE CLONE 120



INTERMEDIATE CLONE 120Exo55

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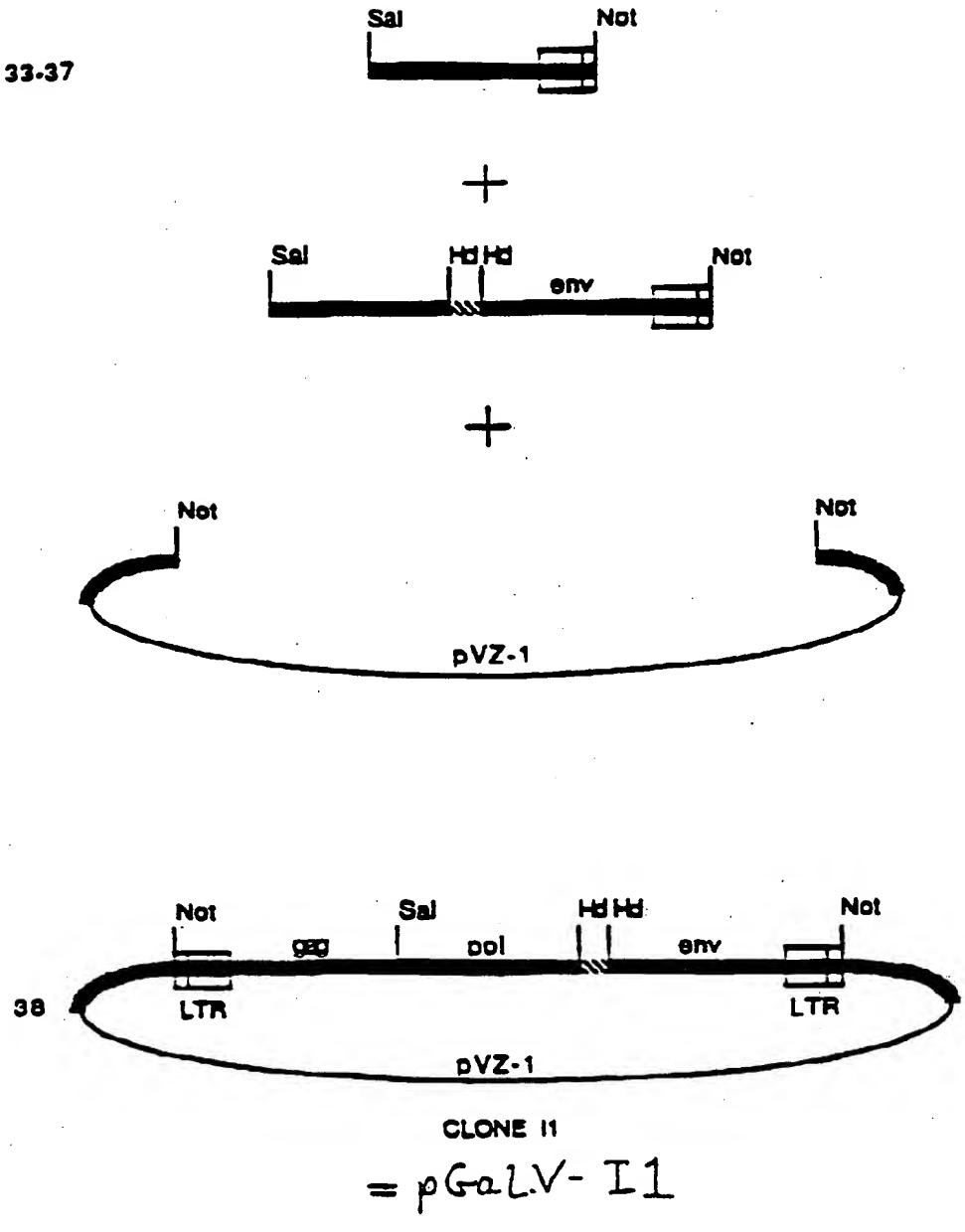
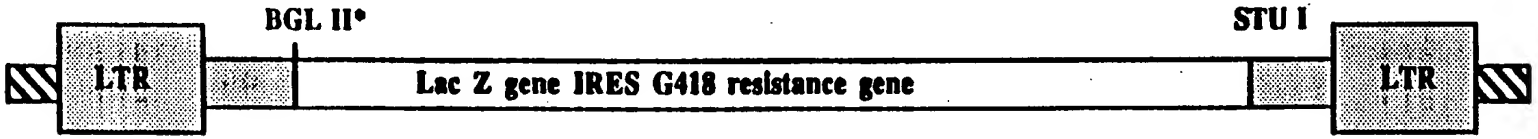
FIGURE 2F COUPLING OF CLONE 66Exo52 INSERT AND CLONE 120Exo88 INSERT: SEPARATION OF LTR'S AND GENERATION OF POTENTIAL INFECTIOUS CLONE



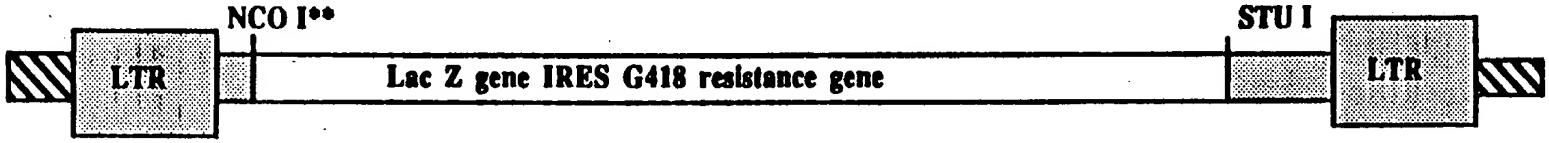
CLONE 11

= pGaLV-I1

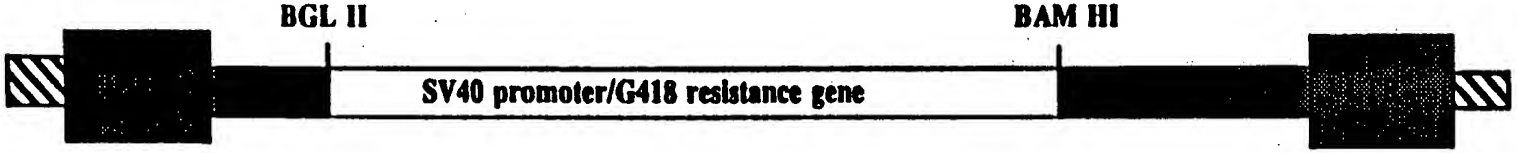
2. Plasmid 2
Packagable GaLV SEATO construct I



3. Plasmid 3
Packagable GaLV SEATO construct II



4. Plasmid 4
Packagable GaLV SF construct I



*nucleotide 1290 in paper by Delassus et al. 1989
**nucleotide 910 " "

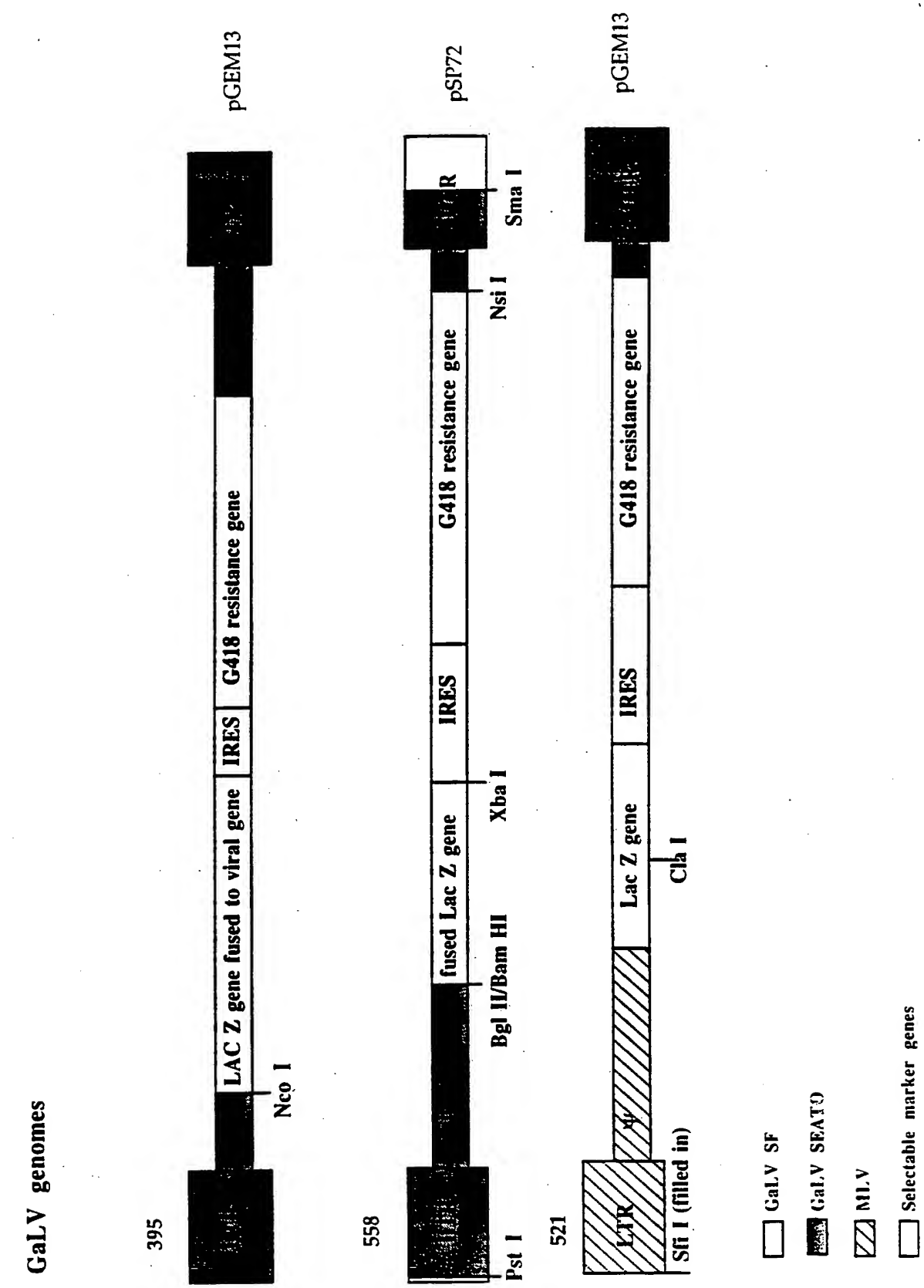
GaLV SF

GaLV SEATO

Selectable marker genes

plasmid sequences

Figure 3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LAC Z gene fused to viral gene
IRES
G418 resistance gene
Nco I
pGEM13
558
fused Lac Z gene
IRES
G418 resistance gene
R
Pst I
Bgl II/Bam HI
Xba I
Nsi I
Sma I
pSP72
521
LTR
Ψ
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IRES
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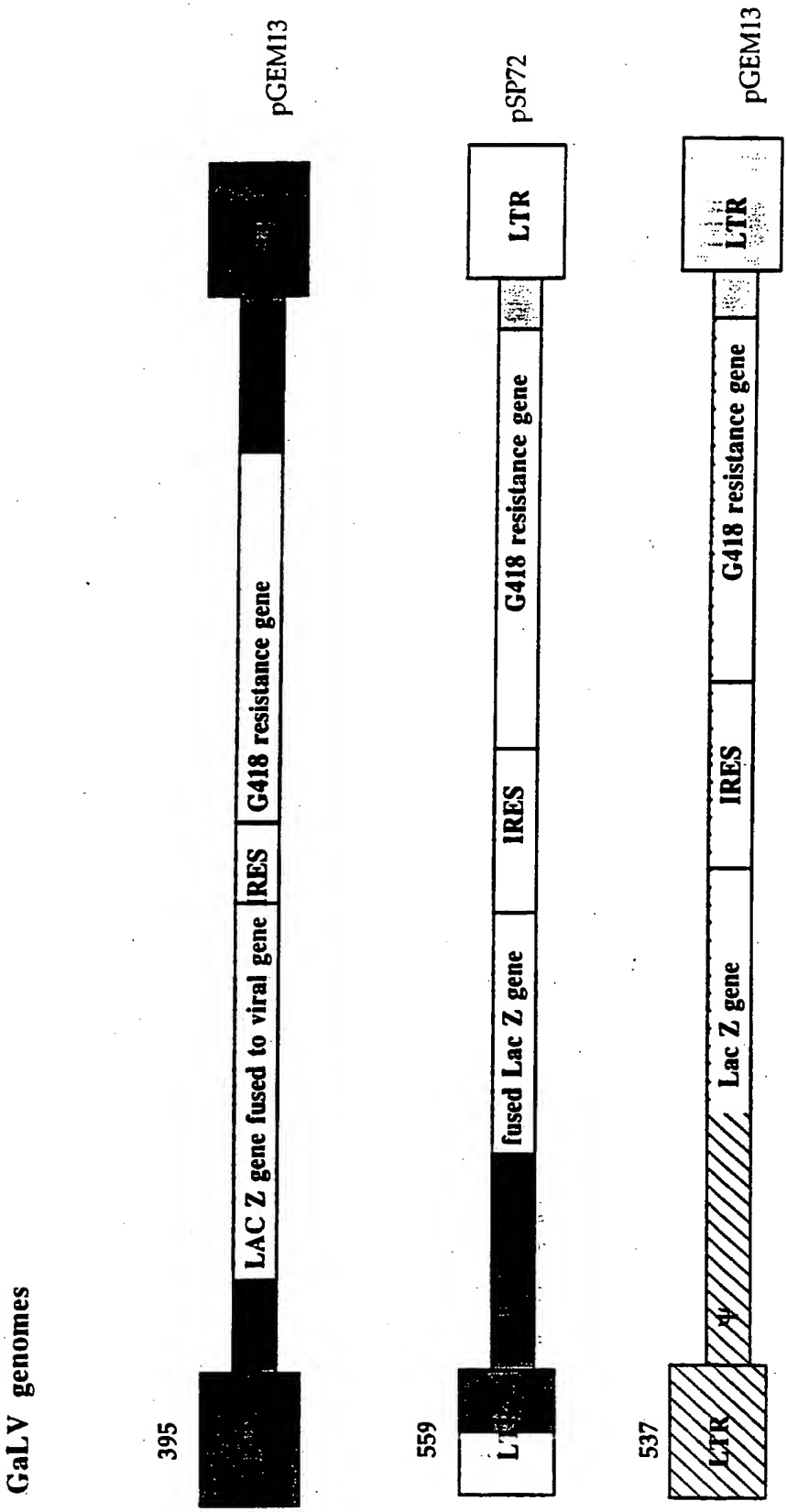
Cla I
pGEM13
GaLV SF
GaLV SEATO
MLV
Selectable marker genes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LAC Z gene fused to viral gene
RES
G418 resistance gene
pGEM13
559
fused Lac Z gene
IRES
G418 resistance gene
LTR
pSP72
537
LTR
Lac Z gene
IRES
G418 resistance gene
LTR
pGEM13
GaLV SF
GaLV SEATO
MLV
Selectable marker genes